A disease associated mutation in fibrillin-1 differentially regulates integrin mediated cell adhesion

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Running title: *Fibrillin-1 mutation differentially affects integrin binding*

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ABSTRACT

Fibrillins serve as scaffolds for the assembly of elastic fibers that contribute to the maintenance of tissue homeostasis and regulate growth factor signaling in the extracellular space. Fibrillin-1 is a modular glycoprotein that includes 7 latent transforming growth factor beta (TGFβ) binding protein-like (TB) domains and mediates cell adhesion through integrin binding to the RGD motif in its 4th TB domain. A subset of missense mutations within TB4 cause Stiff Skin Syndrome (SSS), a rare autosomal dominant form of scleroderma. The fibrotic phenotype is thought to be regulated by changes in the ability of fibrillin-1 to mediate integrin binding. We characterized the ability of each RGD-binding integrin to mediate cell adhesion to fibrillin-1 or a disease-causing variant. Our data show that 7 of the 8 RGD-binding integrins can mediate adhesion to fibrillin-1. A single amino acid substitution responsible for SSS (W1570C) markedly inhibited adhesion mediated by integrins α5β1, αvβ5, and αvβ6, partially inhibited adhesion mediated by αvβ1 and did not inhibit adhesion mediated by αvβ3, α8β1 or αIIbβ3. In the SSS mutant background, introduction of a new cysteine residue in place of a highly conserved tryptophan-1570 alters the conformation of the region containing the exposed RGD sequence within the same domain to differentially affect fibrillin's interactions with distinct RGD-binding integrins.

INTRODUCTION

Fibrillins, large, multi-domain glycoproteins, are central organizers of elastin-containing microfibrils and serve as scaffolds for the assembly of multi-protein complexes that contribute to the maintenance of tissue homeostasis and the regulation of growth factor signaling in the extracellular space (1). Three different variants of fibrillin are present in humans; fibrillin-1 (FBN1), fibrillin-2 (FBN2) and fibrillin-3 (FBN3) (2). All three variants are ~350 kDa proteins that are structurally related to the latent TGFβ-binding proteins (LTBPs). Fibrillin-1 is the major component of tensile-strength transmitting 10-12 nm microfibrils of the ECM and many mutations in the *FBN1* gene cause Marfan syndrome (3-5). Fibrillin-2 has been shown to play a more primary role in the formation of microfibrils during embryonic development and mutations in this variant lead to congenital contractural arachnodactyly (6). Much less is known about fibrillin-3; like fibrillin-2, it's expression pattern is highest in fetal tissues and it localizes predominately to the brain (7).

The genomic organization of fibrillin-1 was originally described by Pereira et al in 1993 (8). Fibrillin-1 was shown to be structurally composed of 5 distinct domains. Like the LTBPs and many other extracellular and cell surface proteins, fibrillin-1 contains a large number of cysteine rich sequences that are homologous to epidermal growth factor (EGF). These EGF-like domains compose approximately 75% of the protein and of the 47 EGF-like domains, 43 are calcium binding (cbEGF) (9). Fibrillin-1 also contains 7 TGFβ binding protein-like domains (TB) that are similar to domains found in the LTBP family (10). The remaining domains exist at lower frequency; a fibrillin unique N-terminal (FUN) domain, a proline rich domain and 2 hybrid domains that share similarities with both the EGF-like and TB domains (11).

The majority of FBN1 mutations have been linked to the development of Marfan syndrome, a connective tissue disorder that results in cardiovascular, skeletal and ocular defects (3,4). However, a subset of missense mutations within a single domain, TB4, result in stiff skin syndrome (SSS), a vastly phenotypically dissimilar disease characterized by short stature, joint stiffness and thickening of the skin (12,13). TB4 is the only domain in fibrillin-1 that contains an exposed arginine, glycine, aspartic acid tri-

peptide (RGD), a common recognition motif for binding a subset of members of the integrin family (14). This observation has led to the suggestion that SSS might be due to altered interactions between fibrillin-1 and one or more integrins (12). Further support for this hypothesis was provided by the observation that mice with a knock-in of the most common SSS disease-inducing mutation, and mice with a presumed loss-of-integrin binding mutation (knock-in of a glutamic acid for aspartic acid in the RGD domain) each developed increases in skin stiffness and thickness reminiscent of human SSS (15).

Prior reports have suggested that three integrins, αvβ3, α5β1 and αvβ6 can bind to the RGD domain of fibrillin-1. The binding capacities of integrins αvβ3 and α5β1 were determined through cell-based assays measuring adhesion to fibrillin-1 or through changes in cell spreading and cytoskeletal rearrangement (13,16, 17). A more quantitative approach using surface plasmon resonance (SPR) analysis by Jovanovic et al in 2007 concluded that αvβ6 can also bind fibrillin and has a low k_d value for fragments containing the TB4 domain (18,19). However, there are 8 well-characterized RGD-binding integrins; αvβ1, αvβ3, αvβ5, αvβ6, αvβ8, α5β1, α8β1 and αIIbβ3 (20). The relative effectiveness of each of these integrins to bind fibrillin-1, and the effects of disease causing mutations on interactions with the full range of fibrillin-1 binding integrins has not been systematically evaluated.

In this paper, we developed cell-based assays to systematically study the ability of each of the RGD-binding integrins to mediate cell adhesion to fibrillin-1. We further sought to determine how the most common SSS missense mutation in the TB4 domain might differentially affect binding to each fibrillin-binding integrin. We found that 7 of the 8 RGD-binding integrins can mediate adhesion to fibrillin 1, but that adhesion mediated by only 4 of these (αvβ1, αvβ5, αvβ6, and α5β1) is affected by the SSS mutation. Our findings thus identify a subset of RGD-binding integrins through which inhibition of binding to mutant fibrillin could contribute to the development of SSS.

RESULTS

Purification of fibrillin-1 fragments from mammalian cells

To determine which members of the RGD-binding integrin subfamily recognize the RGD sequence in fibrillin-1, human recombinant fragments containing domains cbEGF19-cbEGF25, which includes TB4 containing the RGD motif, was purified from HEK293FS cells (Fig 1A). Fragments were purified for the wildtype protein, the SSS disease-causing mutant and the RGE, loss-of-integrin binding control mutant.

Characterization of cell-based systems

We utilized cell adhesion assays to systematically evaluate the ability of the 8 known integrins that bind to RGD-containing ligands to bind to fibrillin-1. For 6 of these, α5β1, α8β1, αvβ3, αvβ5, αvβ6, and αvβ8, we used SW480 cells, a colon cancer cell line that endogenously expresses αvβ5 and α5β1 but does not express any of the remaining RGD-binding integrins (Fig 2A, C). We used transfected SW480 cells expressing individual integrin subunits, β8, β3, β6 or α8, as previously described (22-24), to induce high cell surface expression levels of α8β1, αvβ3, αvβ6 and αvβ8, respectively (Figure 2B). SW480 cells cannot be induced to express functional αvβ1 or αIIbβ3. Integrin αvβ1 was therefore evaluated using human dermal fibroblasts (HDF), which endogenously express functional αvβ1 (Fig 2E). They also express integrins, αvβ3, αvβ5 and α5β1 (Fig 2D). Integrin αIIbβ3 has been shown to be expressed on the cell surface of platelets and was evaluated using human platelets freshly isolated from whole blood (25).

7 of the 8 RGD integrins mediate cell adhesion to wild-type fibrillin-1

To assess the potency of each integrin to individually mediate adhesion to wild-type fibrillin-1 fragments, we isolated the effects of each integrin by blocking every other RGD binding integrin expressed by the utilized cell type and quantified adhesion to dishes coated with a wide range of fragment concentrations (4.5-450 nM). Thus, for wild-type SW480 cells, adhesion mediated by α5β1 was determined in the presence of blocking antibody to αvβ5 (ALULA) and adhesion mediated by αvβ5 was determined

in the presence of blocking antibody to α5β1 (P1D6). For SW480 cells transfected to express additional integrins, adhesion mediated by the heterologously expressed integrin was assessed in the presence of blocking antibodies to both α5β1 and αvβ5 (Table 1). In each panel we compare data for adhesion of the cell line used in the absence of any blocking antibody to adhesion in the presence of blocking antibodies against all other expressed RGD-binding integrins (Figure 3). We found that αvβ5 and αvβ3 were both highly effective receptors for wild-type fibrillin-1 (Fig 3A, B). α5β1 and α8β1 also mediated adhesion, but to a lesser degree (Fig 3C, D). The comparatively weak binding mediated by α5β1 could be explained by the relatively low expression of α5β1 in wild-type SW480 cells. αvβ6 only weakly mediated adhesion, and αvβ8 did not mediate adhesion at all (Fig 3E, F).

Evaluations of integrins αvβ1 and αIIbβ3 were completed using cell adhesion assays with HDF and human platelets, respectively. The individual contribution of αvβ1 was completed using HDF in the presence of blocking antibodies against αvβ3 (AXUM2), αvβ5 and α5β1. The contribution of αIIbβ3 was evaluated using human platelets in the presence of pan-specific αv and β1-blocking antibodies. Both cell types bound well to wild-type fibrillin-1 in the absence of blocking antibodies. Isolation studies with blocking antibodies allowed us to demonstrate that αvβ1 and αIIbβ3 are also highly effective integrin receptors for fibrillin-1 (Fig 3G, H), bringing the total number of integrins that can mediate adhesion to fibrillin-1 to 7. We confirmed that the addition of blocking antibodies specific to the isolated integrin markedly reduced adhesion in every case, demonstrating the specificity of this assay system.

We next determined how the SSS disease causing substitution (W1570C) affected adhesion mediated by each of the 7 integrins identified above. As shown in Figure 4, the disease-causing WC mutation virtually abolished adhesion mediated by αvβ5, α5β1 and αvβ6, but had no effect on adhesion mediated by αvβ3 or α8β1 (Fig 4A-E). In contrast, mutation of the RGD sequence to RGE dramatically inhibited binding of each of these 5 fibrillin-binding integrins. The SSS (WC) mutation substantially inhibited binding of αvβ1 to fibrillin-1, but this inhibition was not complete with adhesion mediated to the WC mutant at an intermediate level between wild-type fibrillin-1 and the RGE mutant (Fig 4F). In contrast,

the WC substitution did not inhibit binding mediated by integrin αIIbβ3. Rather, αIIbβ3-mediated adhesion was modestly increased by the WC mutation. As expected, the RGE mutation did substantially (albeit incompletely) inhibit αIIbβ3-mediated adhesion (Fig 4G).

Taken together, these results suggest that the effects on integrin-binding of the disease-causing WC mutation in fibrillin-1 are not uniform across integrin heterodimers. Although 7 different integrins can bind to the RGD sequence in fibrillin-1 (including 4 that have not been previously identified as fibrillin-1 receptors), only 3 of them, αvβ5, α5β1 and αvβ1 demonstrate binding that is substantially inhibited by the WC mutation.

HDF mediate adhesion to disease-causing fibrillin-1

To further understand the role of the RGD-integrin binding family in the progression of SSS, we used HDFs to assess whether or not the binding capacities determined in the SW480 transfection system were biologically relevant. HDF cells endogenously express αvβ1, αvβ5, αvβ3, and α5β1 (Fig 2D-E). Using the cell adhesion assay system previously described, we were surprised to find notable differences between the binding capacities of integrins expressed on the cell surface of HDFs and the SW480 system. The dermal cell line in the absence of antibody blocking treatment bound to both wildtype and the disease-causing mutant, though its adhesion to the mutant fragment was not as robust. Assays completed in the presence of the RGE-loss of integrin binding control showed no adhesion as expected. Adhesion was also abrogated for cells plated on fibrillin-1 and the disease-causing mutant in the presence of blocking reagents to all four integrins, confirming that HDF adhere to fibrillin-1 specifically through integrin interactions (Fig 5A). In addition to αvβ1, we found that integrins αvβ5, αvβ3, and α5β1 all mediated adhesion to the wildtype fragment as identified with SW480s (Fig 5B-D). However, while αvβ3 and αvβ5 mediated very strong adhesion to fibrillin-1 in SW480 cells (Fig 4A-B), those binding capacities were not reflected in HDFs (Fig 5B-C). Rather, α5β1 (Fig 5D) and αvβ1 (Fig 4F) showed the most robust binding profile to fibrillin-1 in the HDF cell line.

Adhesion assays completed on the disease-causing mutant (WC) confirmed that both α5β1 and αvβ5 are unable to mediate cell adhesion to the mutant fragment (Fig 5B & D). In contrast to the binding profile observed with SW480s, integrin αvβ3 showed complete loss of adhesion in HDFs (Fig 5C). We theorized that this discrepancy may be due to the relative difference in cell surface expression of αvβ3 in the two cell lines (Fig 2B and 2D), as the SW480 over-expression system was designed to introduce high copy number of the transfected integrin subunit. To validate the idea that local concentrations of integrin and ligand can have significant effects on adhesion, we completed additional adhesion assays with the overexpressed SW480-β3 cell line using low concentrations of fibrillin and the disease-causing mutant. When integrin and ligand are present at high concentrations, αvβ3 supports robust adhesion to both the WC mutant fragment and the wild fibrillin-1 fragment (Fig 4B). However, the separation between the binding profiles becomes apparent at ligand concentrations of 5nM or less, suggesting that these interactions are highly dependent on the local stoichiometry of integrin and ligand.

SSS fibrillin-1 mutant contains a free sulfhydryl

The TB4 domain of fibrillin-1 is characterized by an eight-cysteine motif that forms four disulfide bridges responsible for stabilizing the protein fold (Fig 6A). The hydrophobic core of this domain is further stabilized by the presence of tryptophan at position 1570, a feature that is conserved in all TB domains in fibrillins and LTBPs. In the SSS mutant this conserved tryptophan, which in the wild-type structure is within range of three disulfide bonds (Fig 6B), is replaced with a cysteine. In the wild-type structure, the Cβ atom of the Trp-1570 residue is within 5 Å of both Cys-1534 and Cys-1562. This is within the Cβ-Cβ distance seen in disulfide bonds in proteins (26). To determine if the free sulfhydryl remained free or induced dimerization of the mutant fragment, we measured the concentration of protein sulfhydryls using the Ellman's protocol. The Ellman's reagent was replaced with 4,4'-dithiodipyridine (DTDP), an alternative reagent whose small size and amphiphilic nature allow it to react quickly with poorly accessible residues (27). DTDP was reacted with the wildtype and mutant fragment of fibrillin-1 at a range of protein

concentration, from which a concentration of SH was determined. The slope of the resulting plot denotes the number of free sulfhydryls within a protein fragment. The low value of wild-type fibrillin-1 confirms that all the cysteine residues in the protein fragment are sequestered in disulfide bonds (Fig 6C). The SSS mutant, however, showed an increased slope close to the value of 1, suggesting that the mutant maintains a single free sulfhydryl without forming protein dimers. We further confirmed this observation by labeling the two proteins fragments with Alexa-Fluor^{TM} 647 C_2 maleimide, a fluorescent dye that reacts specifically with thiol groups (Fig 6D) and by Coomassie staining the fragments in reduced versus non-reduced conditions (Fig 6E). Reaction of the mutant fibrillin-1 fragment with the fluorescent dye confirmed the presence of an additional free cysteine residue while the Coomassie staining demonstrated that both fragments remain monomers in solution.

The cell adhesion data strongly suggest that the SSS mutation does not simply eliminate integrin interactions with the RGD sequence in the TB4 domain. Introduction of a free sulfhydryl does not cause the protein to dimerize but instead induces a subtle change in the conformation of the RGD site that specifically, and differentially, mitigates interaction with only a subset of fibrillin-1-binding integrins.

Discussion

The results of this study show that 7 of the 8 RGD binding integrins can mediate cell adhesion to wild-type fibrillin-1. Four of these, α8β1, αvβ1, αvβ5 and αIIbβ3 have not be previously described as receptors for fibrillin-1. Mutation of the RGD sequence in the fourth TB domain to RGE abrogated adhesion mediated by 6 of these integrins (α5β1, α8β1, αvβ1, αvβ3, αvβ5 and αvβ6) and substantially inhibited adhesion mediated by αIIbβ3, confirming that cell adhesion was mediated by direct interaction of each of these integrins with the RGD site. In contrast to the RGE mutation, the tryptophan to cysteine missense mutation responsible for causing SSS differentially affected binding of these integrins to fibrillin-1. Adhesion mediated by α5β1, αvβ5 and αvβ6, at the levels expressed on the respective cell lines, was entirely abrogated by the disease-causing mutation and adhesion mediated by αvβ1 and αvβ3 was partially

abrogated. In contrast, adhesion mediated by α8β1, and αIIbβ3 was either unaffected or modestly enhanced (in the case of αIIbβ3).

Progression of SSS has been studied extensively by histologic evaluation of the dermis of affected patients. Immunohistochemistry of skin biopsies suggests that dermal fibroblasts are responsible for the increase in collagen that is observed in patients (12). The adhesion assays completed in human dermal fibroblasts allowed us to use a biologically relevant cell line to evaluate the integrins that could play a role in disease progression, namely αvβ3, αvβ5, α5β1 and αvβ1. While integrins αvβ5 and α5β1 behaved similarly in both HDF and SW480 cell lines, αvβ3 showed robust binding to the mutant fragment in the transfected SW480 cells that was not observed in HDFs. While surprising, this discrepancy suggests that the local concentration of integrin and ligand plays a critical role in how cells respond to the disease-causing mutation in fibrillin-1. The effect was emphasized by the finding that in SW480 cells overexpressing β3, adhesion to the wildtype fragment persists at low nM concentration while adhesion to the mutant fragment begins to diminish significantly. This difference suggests that magnitude of the impact of the W to C mutation on cell adhesion mediated by RGD-binding integrins can be substantially impacted by the local stoichiometry of integrin and ligand.

A similar observation was also made for αvβ5 and α5β1. While the dramatic impact of the W to C mutation on cell adhesion mediated by each integrin was consistent between cell lines, the relative importance of each integrin in adhesion to the wildtype fragment differed substantially. Integrin αvβ5 was the dominant mediator of adhesion to fibrillin-1 in SW480 cells, whereas in HDF α5β1 was dominant. This difference is likely explained by the higher level of expression of α5β1 in HDFs. The differences observed between these two cell lines highlight the importance of gaining a clearer understanding of the local cellular and matrix composition of the dermis in SSS.

As noted earlier, mice expressing either a knock-in of a cysteine for tryptophan substitution in mouse fibrillin-1, at the same location as the human disease-causing WC mutation, or a knock-in of a glutamic acid for aspartic acid substitution in the exposed RGD site (RGE), each develop skin thickening

reminiscent of stiff skin syndrome (14). These results strongly support the hypothesis that SSS is due, at least in part, to loss of integrin binding to mutant fibrillin-1. Our results using the HDF cell line suggest that integrins α5β1, αvβ3, αvβ1 and αvβ5 are each candidates to contribute to disease progression.

It has been suggested that cells plated on the mutant fragment show a different cell surface expression profile for several relevant integrins (15). Increases in active αvβ3 and α5β1 have also been observed through studies of the Fbn1$^{W1572C/+}$ mouse model. These differences were observed in dermal cell lines as well as infiltrating dendritic cells, suggesting that the composition of the local matrix could affect cell surface integrin expression or activity. Fbn1$^{W1572C/+}$ mice haploinsufficient for the β3 subunit were protected from progression of skin thickening and stiffness (15). Our results demonstrating that αvβ3 is capable of mediating adhesion to the mutant fragment at high cell surface expression levels raise the possibility that signals transmitted by mutant fibrillin through αvβ3 could contribute to SSS pathology.

In wild-type fibrillin-1, the fragment used for cell adhesion assays contains an even number of cysteine residues that are all included in disulfide bonds, leaving no free sulfhydryls, as confirmed by fluorescent labeling and DTDP reactivity. In the absence of extensive secondary structure and an extended hydrophobic core, the individual domains of fibrillin-1 rely on a large number of disulfides for structural stability. The mutation of a highly conserved tryptophan to cysteine within the RGD-containing TB4 domain could easily prompt disulfide shuffling given its key role in stabilizing the hydrophobic core of this domain. This large hydrophobic residue (Trp-1570) also plays a prominent role in packing against adjacent disulfide bonds, and hence in stabilizing their pairing. The WC mutation removes the tryptophan that forms the hydrophobic core of domain TB4 and replaces it with an additional cysteine, which could remain as a free sulfhydryl, initiate dimerization of the fragment or cause a misfold with a free thiol that is inaccessible. Coomassie staining of the mutant in non-reduced conditions, as well as reactivity with DTDP, confirmed the presence of a single cysteine which remained free but did not result in protein dimerization. Since the eight-cysteine motif of TB4 is responsible for stabilizing the globular structure of this domain, the addition of a new cysteine residue at position 1570 has the potential to change the stability of the mutant fragment

by causing the reshuffling of disulfide bonds and creating a free sulfhydryl at a separate location. In either case, this mutation is likely to cause a significant change in the conformation of the TB4 domain, affecting the structural context of the exposed RGD site.

Rearrangements in the internal conformation of this domain would lead to alterations in tertiary structure, which would be expected to influence the specificity and affinity of RGD-binding integrins for this domain. While these integrins interact with the local RGD site, it is well known that different RGD-binding integrins preferentially interact with RGD sites depending on the surrounding sequences and on the secondary and tertiary structures of ECM proteins (28-29).

A functional RGD sequence is defined by a flexible loop that allows for a conformation in which the side chains of the tripeptide are pointed out in almost opposite directions (30-31). The outward extension of the motif allows for ease of accessibility by RGD-binding receptors; tertiary or quaternary structures that result in the burial of the RGD sequence will not interact with the integrin binding pocket. Furthermore, the amino acids flanking the C-terminus of the RGD-domain have been shown to have a significant effect on the ability to mediate robust integrin binding. Peptides containing glycine, tryptophan or phenylalanine following the RGD motif are easily recognized by $\alpha5\beta1$ (32). Substitution of serine or alanine in those sites results in preferential binding by $\alpha v\beta3$ and $\alpha v\beta5$. An even more specific peptide containing tyrosine or arginine results in highly potent recognition by $\alpha IIb\beta3$ (33). Not only is the immediate flanking sequence of the RGD motif important for integrin binding, the positioning of a ligand's secondary structure can also have a dramatic effect. For example, integrins $\alpha5\beta1$ and $\alpha v\beta3$ are both known receptors for mediating cell adhesion to fibronectin (FN). While $\alpha v\beta3$ recognizes the RGD motif in the 10^{th} type III repeating unit of FN, $\alpha5\beta1$ requires a synergy site in the 9^{th} type III unit, PHSRN (34). Absence of this amino acid sequence results in a substantial loss of binding by $\alpha5\beta1$ but does not affect the binding capacity of $\alpha v\beta3$. These differences in recognition demonstrates the importance of FN topology for receptor binding to the RGD site (35-36). Fibrillin binding to $\alpha v\beta3$ and $\alpha v\beta6$ has also been shown to be dependent on domain context. $\alpha v\beta3$ requires cbEGF22 together with TB4 to achieve high affinity binding, whilst $\alpha v\beta6$ does not. (37)

Although the RGD site of fibrillin-1 is 20 Å removed from the mutated tryptophan-1570 site, it is reasonable to expect the W1570C mutation to influence integrin specificity by both altering the local conformational energy and dynamics of its RGD tripeptide as well as well as more distant interactions. Our results further demonstrate the importance of secondary and tertiary structure in ligand binding interactions for the RGD-integrin subfamily. The cell adhesion assays demonstrate that the WC mutant of fibrillin-1 does not simply abrogate binding of the 7 fibrillin-1 integrins. Our data shed light on how the WC mutation in fibrillin-1 could abrogate interaction with a specific subset of integrins, which is presumably the first step in the initiation of the cutaneous pathology that characterizes this disease.

Experimental procedures

Reagents: Purified mouse anti-CD51 (integrin αv) was purchased from BD Biosciences; rabbit anti-integrin β1 was purchased from Millipore; ChromePure Mouse IgG was purchased from Jackson ImmunoResearch. Anti-αv mouse monoclonal antibody, L230 (38), was purified in our lab from a hybridoma obtained from ATCC. Anti-α5β1 mouse monoclonal antibody, P1D6 was a generous gift from Elizabeth Wayner (Fred Hutchinson Cancer Center). Anti-αIIbβ3 mouse monoclonal antibody, 10E5 was a generous gift from Barry S Coller (Rockefeller University). Anti-α8β1 mouse monoclonal antibody, YZ83 was a generous gift from Yasuyuki Yokosaki (Hiroshima University). Mouse monoclonal antibodies against αvβ5 (ALULA), αvβ3 (AXUM2) and αvβ8 (ADWA-11) were generated in our laboratory and anti-αvβ6 mouse monoclonal antibody, 3G9, was a generous gift from Biogen Inc (Table 2). 4,4'-dithiodipyridine was purchased from Sigma-Aldrich and Alexa-Fluor^{TM} 647 C_2 maleimide was purchased from Invitrogen. Compound C8 was synthesized by in Dr. Hynil Jo in the Department of Pharmaceutical Chemistry at UCSF and resuspended in 50% DMSO.

Cells: Human dermal fibroblasts (HDF) and growth kit medium were purchased from ATCC and cultured according to the vendor's instructions. Colon carcinoma SW480 cells were used to generate transfected cell lines that overexpressed integrin subunits, α8, β3, β6, and β8, as previously described.

Isolation of human platelets: Whole blood was drawn using a plastic syringe containing 1/10 volume CPD buffer (15 mM citric acid, 90 mM sodium citric, 16 mM $Na_2H_2PO_4$, 142 mM D-glucose, pH 7.4) with 1 μM PGE-1. Cells were mixed gently, transferred to a 50 mL conical and spun at 200xg for 15 min, room temp. The top layer containing the platelet-rich-plasma was transferred to a new 15 mL conical containing 1/10 volume of buffer ACD (39 mM citric acid, 75 mM sodium citric, 135 mM D-glucose, pH 7.4). PGE-1was added to a final concentration of 0.4 μg/mL to prevent platelet activation. Cells were pelleted by centrifugation at 800xg for 20 min at room temp. Platelets were rinsed with wash buffer (10 mM sodium citric, 150 mM NaCl, 1 mM EDTA, 1% D-glucose, pH 7.4) without resuspension to avoid unnecessary activation. Cells were carefully resuspended in 5-10 mL of HEPES-Tyrodes's buffer (134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM dibasic Na_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$ 10mM HEPES, 5 mM glucose, 1% BSA, pH 7.4). For use in adhesion assays, platelets were activated using PAR-1 at a final concentration of 100 μM.

Immunoprecipitation: HDF and SW480 cells were lysed in 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl and 1% Triton X-100, with protease and phosphatase inhibitor cocktail (ThermoScientific). Lysates were centrifuged and total protein concentration determined using Pierce BCA Protein Assay Kit. Samples were incubated with integrin αv antibody L230 for 1 hr at 4°C with rotation (70 rpm). Protein G Sepharose (GE Healthcare) beads were then added with a further 1 hr incubation. Samples were washed four times with 1 mL lysis buffer and eluted with reducing sample buffer, resolved by SDS-PAGE, and analyzed by immunoblot.

Flow cytometry: HDF and SW480 cells were collected from 10 cm dishes with 0.05% Trypsin-EDTA and washed twice with PBS. 1 x 10^6 cells were re-suspended in PBS supplemented with 1% BSA and then incubated with primary antibody at 4°C for 1 hr. Cells were washed twice with PBS before incubation with secondary antibody conjugated to phycoerythrin (PE; Jackson ImmunoResearch). Cells were washed twice with PBS before analysis on a BD FACSCantoll. Antibodies were used at a final concentration of 1 μg/mL as follows: anti-integrin β3 (AXUM2), anti-integrin β6 (3G9), anti-integrin β5 (ALULA) and anti-integrin

αv (L230). A chicken anti-integrin α8 (YZ83) antibody was used at 2 μg/mL (generous gift from Dr. Yasuyuki Yokasaki) and anti-integrin α5 (P1D6) was used at 1 μg/mL.

Cell adhesion assay: 96-well flat-bottomed Immulon 4HBX microtiter plates (ThermoScientific) were coated with a series of fibrillin-1 protein concentrations diluted in DPBS and then incubated at 37° C for 1 hr. Wells were washed with DPBS before blocking with 2% BSA prepared in DPBS at 37° C for 1 hr. Cells were detached from confluent 10 cm dishes using 0.05% Trypsin-EDTA and re-suspended in serum-free DMEM. Wells were plated with 5 x 10^4 cells. For blocking conditions, cells were incubated with 10 μg/mL of indicated antibody or 10 μM of C8 (Table 1) for 10 min at 4°C before final plating. Plates were centrifuged at 300 rpm for 5 min prior to a 1 hr incubation at 37°C in a 5% CO_2 humidified incubator. Non-adherent cells were removed by centrifugation (top side down) at 500 rpm for 5 min. Remaining adhered cells were stained with 0.5% crystal violet, 0.1% formaldehyde and wells subsequently washed with PBS. The relative number of cells in each well was determined after solubilization in 40 μL of 2% Triton X-100, absorbance was read at 595 nm in a microplate reader (Bio-Rad Laboratories). All determinations were carried out in triplicate.

Expression and protein purification: The pSecTag2A plasmids containing cbEGF19-cbEGF25 of wild-type fibrillin-1 and the disease-causing mutation (WC) were generously provided by Dr. Penny A. Handford. Mutagenesis of this plasmid was completed using QuickChange Site-Directed Mutagenesis Kit to generate the RGE fibrillin-1 fragment. Nucleotide 1543 was mutated to generate a D to E substitution. All three protein fragments contained a His-tag for purification on the C-terminus and were expressed in HEK293FS cells; a 1 L volume of 1 x 10^9 cells were transfected using 1 mg of DNA (293Fectin™, ThermoScientific). Cells were allowed to grow for 5 days before medium was collected and diluted 3-fold in 50 mM Tris, pH 8, 200 mM NaCl (resuspension buffer) in a Schott bottle. Ni^{2+}-charged chelating sepharose beads (ThermoScientific) were prepped in resuspension buffer before adding to diluted medium and incubated at 4°C for 4 hr with gentle shaking. Sepharose beads were packed into a 10 mL column, washed with 50 mL of cold wash buffer (10 mM imidazole, 50 mM Tris pH 8, 200 mM NaCl). Protein was

eluted with 20 mL of cold elution buffer (300 mM imidazole, 50 mM Tris pH 8.0, 200 mM NaCl). 1 mL fractions were collected and run on 10% SDS-PAGE to determine elution of protein. Fractions were dialyzed at 4°C into resuspension buffer using a Slide-A-Lyzer Dialysis Cassette (Pierce). Final protein concentration was determined using a BCA Protein Assay Kit (Pierce) and fragments were stored at 4°C in 0.1% sodium azide.

Quantification of protein thiols with DTDP: Protein samples were adjusted to a volume of 250 μL in DPBS containing 2% SDS and denatured for 15min. Samples were added to 2.5 mL of reaction buffer (100 mM NaH_2PO_4, 1 mM EDTA, pH 8). Samples were vortexed and incubated at room temp for 5 min after the addition of 50 μL 4 mM DTDP. Absorbance at 324 nm was read against a water blank (1 cm light path). Absorbance values were corrected using an additional DTDP blank. The amount of free sulfhydryl from solution in the spectrophotometric cuvette was calculated according to E = (A)/ bc where A = absorbance, b = path length in centimeters and c = concentration in moles/liter of SH. The molar extinction coefficient for DTDP in this buffer system is 21,400 M^{-1} cm^{-1}. Final concentrations were determined by adjusting for the dilution factor of the 2.8 mL reaction volume.

Fluorescent labeling with Alexa-Fluor™ 647 C_2 maleimide: Protein samples were diluted to 0.2 mg/ml in 100 mM Tris·HCl, pH 7. Alexa-Fluor™ 647 C_2 Maleimide was added to a final concentration of 0.5 mM and samples were incubated in the dark at room temp for 30-60 min. Labeled fragments were run on a 4-12% Tris-glycine SDS-PAGE and imaged using the 650-laser line.

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The abbreviations used are: TGFβ, transforming growth factor-β; SSS, Stiff Skin Syndrome; TB4, TGFβ-binding protein-like domain 4; FBN, fibrillin; RGD, arginine, glycine, aspartic acid tri-peptide; EGF, epidermal growth factor-like; LTBP, latent TGFβ binding protein; SPR, surface plasmon resonance; HDF, human dermal fibroblasts; DTDP, 4,4'-dithiodipyridine; ECM, extracellular matrix